

CLAIMS

What is claimed is:

Claim 1 - Process for determining the primary structure of the messenger RNA coding for the recombinant endooligopeptidase A (EOPA) and its protein sequence, in particular from human, characterized by the following steps:

- a) isolating total RNA from several animal tissues, specifically human, using the guanidine isothiocyanate-phenol-chloroform extraction method or alternatively specific reagents such as the Trizol® (GibcoBRL);
- b) purifying the messenger RNA from total RNA using pre-packed oligo-dT cellulose columns or the resin in suspension;
- c) analyzing the quality of the obtained messenger RNA by electrophoresis in denaturing agarose gel (containing 1-2.5% of formaldehyde), followed by staining with ethidium bromide or with others nucleic acid dyes, photodocumentation, and analysis by hybridization (Northern Blot analysis);
- d) reverse-transcription of the obtained messenger RNA and synthesis of the complementary DNA strand;
- e) cloning of the obtained double-strand cDNA into a plasmid vector, cosmid or phage vector to allow its "in vitro" amplification;
- f) identifying and selecting the EOPA cDNA by hybridization, immune-selection using a specific anti-EOPA antibody or, alternatively, by detection of EOPA specific activity;
- g) amplifying and isolating the target cDNA by PCR ("polymerase chain reaction"), or alternatively by

amplifying the bacteria transformed with the vector containing this cDNA insert, in bacteria, followed by digestion with appropriate restriction enzymes in order to isolate the cDNA insert.

5 **Claim 2 - Process** of claim 1, wherein the nucleic acid dyes described in the step (c) is the "Syber Green" (Molecular Dynamics).

Claim 3 - Process of claim 1, wherein the amplification of the cDNA described in the step (f) is obtained by
10 amplifying the bacteria transformed with the vector containing the cDNA coding for EOPA.

Claim 4 - Process of claim 1, wherein the amplification of the cDNA insert described in the step (f) is obtained alternatively by direct PCR amplification, using total or
15 messenger RNA and specific oligonucleotides, by RT-PCR (reverse-transcription polymerase chain reaction).

Claim 5 - Process of claim 1, wherein the identification and selection of the EOPA cDNA, as described in the step (e), is performed by hybridization or, alternatively, by
20 immune-selection using a specific anti-EOPA antibody, characterized by the following steps:

a) amplification of the cDNA library and transferring the genetic and/or protein material of each individual clone to nitrocellulose membranes by
25 capillarity;

b) hybridization assays using the membranes obtained in the last step and probes, labeled radioactively or not, or alternatively identification of the positive clones using specific antibodies;

c) identification of the positive clones for the employed probes or antibodies, and collection of these isolated clones for later amplification and sequencing of the respective cDNA insert.

5 **Claim 6 - Process** of claim 1, wherein the fragment of about 600 base pairs, as described in the step (f), obtained by digestion of the cDNA coding for EOPA with appropriate restriction enzymes is used for the synthesis of the probes employed for the distribution analysis of the EOPA in brain
10 by "in situ" hybridization.

Claim 7 - Process of claim 6, wherein the restriction enzymes employed are EcoRI and XbaI.

Claim 8 - Process, wherein the cDNA insert, obtained as described in the claim 1 or 2, was completely sequenced in
15 order to determine the respective nucleotide and deduced amino acid sequence, which primary and secondary structure was also analyzed; furthermore, the determined sequence was also used in the search for homologous sequences deposited in specialized data banks.

20 **Claim 9 - Process**, as described in the claim 1 to 8, characterized by the fact that it is particularly employed for the determination of the nucleic primary structure and the respective protein from mammals.

Claim 10 - cDNA insert coding for EOPA, characterized by
25 the fact that it comprises a fragment of about 600 base pairs.

Claim 11 - cDNA insert, as described in the claim 10, characterized by the fact that the complete sequencing of the cDNA insert allows the determination of the nucleic

primary sequence and the respective amino acid deduced sequence, which permit the analysis of the primary and secondary structure of this protein sequence.

5 Claim 12 - cDNA insert, as described in the claims 10 and 11, characterized by the fact that the complete sequencing of the full-length allows the analysis of the homology with other sequences deposited in specialized data banks.

10 Claim 13 - Use of the cDNA insert of claim 10, characterized by the fact that it was used for the synthesis of the probes employed in the distribution analysis of EOPA in brain by "in situ" hybridization.

15 Claim 14 - Process for the production of the human recombinant EOPA, using the cDNA isolated and identified according to the claim 1, specifically using bacteria, wherein the process comprises the steps of:

- 20 a) subcloning of the identified and isolated cDNA coding for EOPA, in frame, into the expression vector, such that EOPA is produced as a recombinant molecule or as a fusion proteins linked to anchors proteins or oligopeptides;
- 25 b) transforming the host bacteria (*Escherichia coli* strains DH α or BL21(DE3) or similar) with the plasmidial construct described above, that is, the expression vector containing the cDNA insert coding for the EOPA cloned in frame, as described in step (a);
- 30 c) amplifying the transformed bacteria by growth in appropriate culture medium, such as LB medium containing antibiotics for selection, until obtaining optic density of about 0.6 for readings at wavelengths between 560 and 600 nm;

- 5 d) production of the target protein by addition of inducing agents such as the IPTG (isopropyl-thiogalactosidase) to the final concentration of about 0.5 to 1 mM, followed by incubation at 30°C or 37°C, with shaking;
- 10 e) collecting the bacteria containing the target expressed protein by centrifugation, and storage at -20°C or immediate processing, being lyzed either by sonication or by mechanical pressure, to release the recombinant or the fusion protein present in the bacteria cytoplasm;
- 15 f) recovering and purifying the fusion protein using affinity columns, electrophoresis, or liquid-phase chromatography;
- 20 g) evaluating the purity and the quality of the recombinant protein by electrophoresis in polyacrylamide gels (SDS/PAGE), HPLC with an UV/vis detector, Western Blotting, mass spectrometry, and/or determining the specific activity, by enzymatic kinetics, using natural and/or synthetic peptide substrates.

25 **Claim 15 - Process**, as described in the claim 14, wherein the identified and isolated cDNA coding for EOPA described in the step (a) is obtained as described in the process of claim 1 to 9.

30 **Claim 16 - Process**, as described in the claim 14, wherein the subcloning described in the step (a) allows the expression of the recombinant EOPA or the EOPA in the fusion form with an anchor protein or polypeptide, which facilitate its purification.

Claim 17 - Process as described in the claim 14, characterized by the fact that in the step (b) the employed host bacteria (*Escherichia coli*) is the strain DH α or BL21(DE3) or similar.

5 Claim 18 - Process as described in the claim 14, characterized by the fact that in the step (c) the employed host bacteria (*Escherichia coli*) is the strain DH α or BL21(DE3) or similar.

10 Claim 19 - Process as described in the claim 14, characterized by the fact that in the step (c) the employed culture medium is the LB containing the antibiotic for selection.

15 Claim 20 - Process as described in the claim 14, wherein the induction of the expression described in the step (d) is done by addition of the inducing agent IPTG (isopropyl-thiogalactosidase).

20 Claim 21 - Process as described in the claim 14, characterized by the fact that in the processing of the bacteria pellet, as described in the step (e), the rupture of the bacteria is performed by sonication or mechanical pressure, in order to release the recombinant or the fusion protein present in the bacteria cytoplasm; the expressed protein can be purified by using affinity columns, electrophoresis or liquid-phase chromatography.

25 Claim 22 - Process as described in the claim 14, wherein the bacteria described in the step (e) are *Escherichia coli* strain DH α or BL21(DE3) or similar.

Claim 23 - Process as described in the claim 14, wherein the transformation of the host cells can be performed by

heat-shock or electroporation of the bacteria, previously and properly, prepared for each of these methods.

Claim 24 - Process as described in the claim 14, wherein the recombinant protein expressed as a fusion protein can be separated from other bacterial soluble proteins (contaminants) using affinity columns containing immobilized nickel, for poly-histidine fusions, or glutathione-sepharose resin, for fusions with GST (glutathione-S-transferase).

Claim 25 - Process as described in the claim 24, wherein the recombinant protein expressed as a fusion protein can be separated from the anchor protein or polypeptide by digestion with proteases, which consensus recognition sequences had been previously inserted between the anchor and the recombinant protein.

Claim 26 - Process as described in the claim 25, wherein the employed proteases are the thrombin, factor X, and enterokinase.

Claim 27 - Process for the determination of the EOPA gene primary structure, wherein the EOPA gene is formed by 9 exons and 10 introns, comprising about 40 kb, located at the chromosome 17p12.9, relatively close to the p53 locus (about 0.7 cM distant), at the same chromosome as the Lisl gene (17p13.3); some recognition sites for transcriptional regulatory factors were observed in the promoter region of the gene.

Claim 28 - Process as described in the claim 27, wherein the putative binding sites for transcription regulatory factors, such as the AP1, cMyb, SP1, nMyc, cMyc, among

others, were identified in the upstream region of the EOPA gene, including the promoter region.

5 **Claim 29 - Process** as described in the claim 27, wherein the complete sequence of the human EOPA gene was determined by the analysis of the human genome complete sequence accessible at specialized data banks.

10 **Claim 30 - Process** as described in the claim 27 to 29, wherein the complete primary structure of the human EOPA gene was determined by the processes described in the claims 1 to 9.

15 **Claim 31 - Human EOPA gene**, characterized by the fact that it is formed by 9 exons and 10 introns, comprising about 40 kb, located at the chromosome 17p12.9, relatively close to the p53 locus (about 0.7 cM distant), at the same chromosome as the Lis1 gene (17p13.3); some recognition sites for transcriptional regulatory factors were observed in the promoter region of the gene.

20 **Claim 32 - Gene** as described in the claim 31, characterized by the fact that the transcription regulatory factors are AP1, cMyb, SP1, nMyc, cMyc, among others.

Claim 33 - Gene as described in the claims 31 and 32, characterized by the fact that it is related to the primary structure of human EOPA.

25 **Claim 34 - Process** for isolation, purification and determination of the amino acid sequence of the natural EOPA, wherein the method comprises the following steps:

- a) homogenization of fresh tissue in 10mM Tris-HCl pH 7.5 buffer, containing 0.25M de sacarose, in the proportion of 1:3 [weight:volume];

- b) ultracentrifugation of the sample at 25,000 xg for 15 minutes, and 100,000 xg for 1 hour;
- 5 c) analysis of the cytosolic fraction (supernatant) by polyacrilamide gel electrophoresis in denaturing conditions (SDS/PAGE), and protein quantification by the method of Bradford or Laemmli, followed by the Western blotting using specific antibodies;
- 10 d) fractionation of the cytosolic fraction by gel filtration chromatography using the Superose column coupled to a liquid chromatography system (Äkta - Amersham Biosciences) using the 20mM Tris-HCl pH 7.4 buffer containing 0.5M of NaCl, at a flow rate of 0.25 ml/min;
- 15 e) determination of the relative molecular weight based on a pre-calibration of the column with standard proteins dissolved in the buffer described above;
- 20 f) identification of the fraction containing the natural EOPA by determination of the specific enzymatic activity fluorimetrically, using quenched fluorescent peptides, or by using HPLC systems and natural peptide substrates;
- 25 g) purification of the natural EOPA using HPLC system coupled to a UV detector system, using the 20mM Tris-HCl pH 7.4 buffer containing 0.5M of NaCl and collecting manually the peaks corresponding to the pure EOPA, which was concentrated by liophylization and analyzed by polyacrilamide gel electrophoresis.

Claim 35 - Process as described in the claim 34, wherein the fresh tissue homogenized in the step (a) is from brain.

Claim 36 - Process as described in the claim 34, wherein the HPLC peak corresponding to the pure natural EOPA observed in the step (g) shows a single band of about 40 kDa.

5 **Claim 37 - Process** as described in the claim 34, wherein the purification of the natural EOPA may be done by using immunoaffinity columns prepared using pre-activated CH sepharose 4b resin for the covalent linkage of the N-terminus of the anti-EOPA antibody.

10 **Claim 38 - Process** as described in the claim 34, wherein the natural pure EOPA may be eluted in its active form by using 2M NaI in 50mM Tris-HCl pH 8.0 buffer, containing 20mM of NaCl.

15 **Claim 39 - Process** as described in the claim 34, wherein the EOPA primary sequence may be determined by mass spectrometry, where the data may first be acquired from the quadrupolo (ES-MS) by scanning the ratio (m/z), and the sequencing may be determined by using a second quadrupolo (ES-MS/MS) by selection of the peptides with a protonated
20 ion characterized in the first quadrupolo, followed by a fragmentation performed by collision induced dissociation (CID).

Claim 40 - Process as described in the claim 34, wherein the mass spectrometer (ES-MS/MS) is operated in the
25 positive ionization mode, equipped with an electrospray ion source and using software for the data acquisition.

Claim 41 - Process as described in the claim 34, wherein the sequencing may be performed by the HPLC system coupled to the mass spectrometer (LC-ESMS/MS).

Claim 42 - Process as described in the claim 34, wherein the amino-terminus of the protein may be sequenced by the Edman degradation method or deduced from amino acid composition analysis.

5 Claim 43 - Amino acid sequence of natural EOPA, characterized by the fact that the primary sequence may be determined by mass spectrometry, where the data may first be acquired from the quadrupolo (ES-MS) by scanning the ratio (m/z), and the sequencing may be determined by using
10 a second quadrupolo (ES-MS/MS) by selection of the peptides with a protonated ion characterized in the first quadrupolo, followed by a fragmentation performed by collision induced dissociation (CID).

Claim 44 - Fraction containing the natural EOPA,
15 characterized by the fact that the HPLC peak corresponding to the pure natural EOPA shows a single band of about 40 kDa.

Claim 45 - Process for the generation of the polyclonal anti-EOPA antibodies, specifically anti-human EOPA
20 polyclonal antibodies generated in mice, which method comprises the following steps:

- a) generating polyclonal antibodies by immunization with natural EOPA purified from animal brain, or using active recombinant protein produced in bacteria;
- 25 b) immunizing Balb-C or High III mice, 7 to 8 weeks old, weighing 18 to 22 g;
- c) performing intradermical injections of 2 µg of the purified protein or 3 µg of the recombinant protein, absorbed on aluminum hydroxide [Al(OH)₃] or on

incomplete Freud's adjuvant, in weekly or monthly intervals for each of the four immunizations;

d) collecting blood samples, one week or one month after the last immunization, and the serum can be stored at -20°C;

e) evaluating the antiserum titer by ELISA and Western blot, using the appropriate antigen;

f) evaluating the anti-EOPA antiserum, using as substrate the natural peptide bradykinin or the fluorescent substrate Abz-GFAPFRQ-EDDnp, whose cleavages are monitored by HPLC and/or by fluorimetry, and verification of the anti-serum ability to block the EOPA peptidase activity.

Claim 46 - Process as described in the claim 45, wherein said the generation of the polyclonal antibodies against EOPA is specific for the anti-human EOPA raised in mice.

Claim 47 - Polyclonal antibodies anti-EOPA, characterized by the fact that they are obtained by the process described in the claims 45 and 46.

Claim 48 - Process for the characterization of the biochemical and proteolytic properties of EOPA, characterized by the fact that kinetic assays using natural peptide and/or synthetic fluorescent substrates was employed.

Claim 49 - Process, as described in the claim 48, characterized by the fact that both the natural and the recombinant protein show exactly the same enzymatic and biochemical characteristics.

Claim 50 - Process, as described in the claim 48, characterized by the fact that the EOPA is an endopeptidase thiol-activated and insensible to EDTA, showing molecular mass of approximately 40 kDa, being found associated with
5 other cytosolic proteins generating complexes of molecular masses higher than 70 kDa.

Claim 51 - Process, as described in the claim 48, characterized by the fact that the natural EOPA and the respective recombinant protein are able to selectively
10 hydrolyze peptides of 7 to 13 amino acid residues, they both have an isoelectric point between 5.22 and 5.50.

Claim 52 - Process described in the claim 48, wherein said both the natural and the recombinant proteins hydrolyze specifically the Phe⁵-Ser⁶ bond of the bradykinin, and the
15 Arg⁶-Arg⁹ bond of the neurotensin, also releasing the [Met⁵]- or [Leu⁵]-enkephalins from several opioid peptides containing enkephalins in their sequences.

Claim 53 - Process described in the claim 48, wherein the substrates of EOPA are several neuropeptides or their
20 derivatives, showing that it is not possible to determine the sub-sites of interaction between the substrate and the enzyme in order to predict the cleavable peptide bond, besides being demonstrated the strict correlation of the size and conformation of the substrate for the proteolytic
25 action of the EOPA.

Claim 54 - Process for identification of EOPA inhibitors, wherein the EOPA is a thiol-dependent enzyme, since it is activated by the reducing agent dithiothreitol (DTT) and inhibited by classic inhibitors of the cysteinyl proteases,
30 such as the thiol compounds.

Claim 55 - Process, as described in the claim 54, wherein the thiol compounds are the *p*-chloro-mercuribenzoate (PCMB) and the 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB).

5 **Claim 56 - Process**, as described in the claims 54 and 55, wherein the use of the mimetic compounds of the substrates of this enzyme, like derivatives of dynorphin, labeled with a thiol-reactive group [Npys] (S-(3-nitro-2-pyridine-sulfenyl), shows the existence of a critical cysteine residue close to the catalytic site, which reacts
10 irreversibly with the site-directed inhibitor.

Claim 57 - Process, as described in the claims 54 and 55, wherein the reactive SH group of the enzyme reacts irreversibly with the thiol-reactive group [Npys] (S-(3-nitro-2-pyridine-sulfenyl) present in the site-directed
15 inhibitor, suggesting the enzyme is a cysteinyl protease.

Claim 58 - Process, as described in the claims 54 and 55, characterized by the fact that EOPA is a thiol-activated endooligopeptidase, insensible to the EDTA.

20 **Claim 59 - Process** as described in the claims 54 and 55, characterized by the fact that EOPA is inhibited by the metalloproteases inhibitors, such as the cFP (N-[1(R,S)-carboxy-3-phenylpropyl]) and the JA2 (N-[1(R,S)-carboxy-3-phenylpropyl]-A- α -aminoisobutiric acid-Y-p-aminebenzoate), which is a derivative synthesized from the cFP specific to
25 the thimet oligopeptidase (TOP).

Claim 60 - Process as described in the claims 54 and 55, characterized by the fact that both compounds (JA2 and cFP) also inhibit the catalytic activity of the EOPA with a similar K_i (~17 nM).

Claim 61 - Process, as described in the claims 54 to 60, characterized by the fact that they are performed based on the process described in the claim 48.

5 **Claim 62 - Recombinant protein**, characterized by the fact that it is an endopeptidase thiol-activated and insensible to EDTA, showing molecular mass of approximately 40 kDa, being found associated with other cytosolic proteins generating complexes of molecular masses higher than 70 kDa.

10 **Claim 63 - Protein**, as described in the claim 62, characterized by the fact that it is able to selectively hydrolyze peptides of 7 to 13 amino acid residues, they both have an isoelectric point between 5.22 and 5.50.

15 **Claim 64 - Protein**, as described in the claims 62 and 63, characterized by the fact that it hydrolyzes specifically the Phe⁵-Ser⁶ bond of the bradykinin, and the Arg⁸-Arg⁹ bond of the neurotensin, also releasing the [Met⁵]- or [Leu⁵]-enkephalins from several opioid peptides containing enkephalins in their sequences.

20 **Claim 65 - Protein**, as described in the claims 62 to 64, characterized by the fact that it is a thiol-dependent enzyme, since it is activated by the reducing agent dithiothreitol (DTT) and inhibited by classic inhibitors of the cysteinyl proteases, such as the thiol compounds.

25 **Claim 66 - Protein**, as described in the claims 62 to 65, characterized by the fact that it shows a critical cysteine residue close to the catalytic site.

Claim 67 - Process for the utilization of the polyclonal antibodies against EOPA, wherein the anti-EOPA antibodies

are specific inhibitors of the EOPA catalytic activity (do not inhibit other peptidases, specifically TOP and NL) and interfere in the process of interaction with oligopeptides and proteins.

5 **Claim 68 - Process** as described in the claim 67, characterized by the fact that it refers to the anti-human EOPA antibody raised in mice, as described in the claim 45.

Claim 69 - Process for the utilization of the polyclonal antibodies against EOPA, wherein the distribution of the
10 EOPA may be determined by quantifying the specific proteolytic activity, immunohistochemistry, "in situ" hybridization and/or Northern blot.

Claim 70 - Process as described in the claim 69, characterized by the fact that it refers to the anti-human
15 EOPA antibody raised in mice, as described in the claim 45.

Claim 71 - Process as described in the claim 69, characterized by the fact that between the analyzed tissues by quantifying the specific proteolytic activity, the kidney cytosol shows the lowest oligopeptidase activity of
20 EOPA; while the thimet oligopeptidase (TOP) is responsible of 60% of the total oligopeptidase activity of this tissue when the substrate Abz-GFAPFRQ-EDDnp is used.

Claim 72 - Process as described in the claim 71, characterized by the fact that the analyzed tissues are
25 brain, testes, heart, spleen, liver, lung, skeletal muscle and kidney.

Claim 73 - Process as described in the claim 69, characterized by the fact that the EOPA and TOP (thimet oligopeptidase) activity being homogeneously distributed in

the cytosol of the analyzed tissues in general manner, except for the brain and kidney.

5 **Claim 74 - Process** as described in the claim 69, characterized by the fact that the EOPA has preferential distribution in the cytosol of the rat brain, presenting 60% of the total oligopeptidase activity.

10 **Claim 75 - Process** as described in the claim 74, characterized by the fact that the thimet oligopeptidase (TOP) activity was not detected in the cytosol of the rat brain.

15 **Claim 76 - Process** as described in the claim 74, characterized by the fact that it was shown that besides EOPA, 20% of the activity of this cytosol is due to the activity of NL, and other 20% are due to the action of other(s) non-identified cytosolic enzymes, which hydrolyzes the substrate.

20 **Claim 77 - Process** as described in the claim 69, characterized by the fact that the distribution of EOPA was also determined by "in situ" hybridization assays, showing that the expression of EOPA is stronger in the brain.

25 **Claim 78 - Process** as described in the claim 69, characterized by the fact that the expression of EOPA is stronger in the brain, showing higher expression level in some layers of the cortex, hippocampus, cerebellum, and the basal nucleus of Meynert.

Claim 79 - Process as described in the claim 69, characterized by the fact that the immunohistochemical studies of tissue and cellular distribution, the co-localization of the EOPA with opioid peptides and their

precursors is identified in the central nervous system, in the cell body and neuronal axons of the vertebrate retina, which are rich in [Leu⁵]-encephalin.

5 **Claim 80 - Process** as described in the claim 69, characterized by the fact that the EOPA is secreted into the extracellular space, in an analogous form to other peptide messenger metabolizing enzymes.

10 **Claim 81 - Process** as described in the claim 69, characterized by the fact that the "in situ" hybridization studies, employing brain slices of newly born rats or embryos, suggest an increase in transcription level of the messenger RNA coding for the EOPA, mainly in the cortex of newly born embryos approximately between days 5 and 10.

15 **Claim 82 - Process for identification of congenital, infectious and degenerative pathologic conditions of the central nervous system,** characterized by the fact that the EOPA has an important role in the process of the central nervous system formation during the embryogenesis.

20 **Claim 83 - Process** as described in the claim 82, characterized by the fact that the identified EOPA presents high evolutionary conservation of the "coiled-coil" domain, and is expressed in different animals, as in human, rabbit, mice and rat.

25 **Claim 84 - Process** as described in the claim 83, characterized by the fact that the identified EOPA is expressed in different animals, and is also called as Nude-L or Nude2.

Claim 85 - Process as described in the claim 83, characterized by the fact that the "coiled-coil" domain of

the EOPA presents a helical structure, and is related to the nuclear and neuronal migration, and has an important function in the process of cellular movement occurring during embryogenesis.

5 **Claim 86 - Process** as described in the claim 84, characterized by the fact that the proteins homologous to the EOPA, isolated from rat (Nude2, GenBank Acc. No. NM_133320) and from mice (Nude-L, GenBank Acc. No. AF323918), also present oligopeptidase activity upon
10 substrate used to characterize the EOPA, besides showing blockage of their proteolytic activity by specific inhibitors of the human, the rabbit and the rat EOPA.

Claim 87 - Process for the determination of the distribution and the role of EOPA in the cultured cells,
15 characterized by the fact that the detection and the determination of the role of the EOPA in cell cultures is performed by assays, which identify the increased expression of the messenger RNA of the EOPA in embryonic corpuscles and in the differentiating cells, around the 8th
20 day after the treatment with retinoic acid, when neurites are formed, and utilize the totipotent p19 cells, derived from a murine embryonal carcinoma, as a model of "in vitro" differentiation and for the identification of the factors regulating the differentiation process triggering.

25 **Claim 88 - Process** as described in the claim 87, characterized by the fact that the EOPA expression may be modulated at genetic and protein levels during the neuronal differentiation process.

Claim 89 - Process, as described in the claim 87,
30 characterized by the fact that the EOPA proteolytic

activity is mainly found in the cytosol of the PC12 cells, derived from a rat tumor (transplantable rat pheochromocytoma), which oligopeptidase activity is modulated by the cAMP and/or by the treatment with compounds such as the FGF or retinoic acid.

Claim 90 - Process as described in the claim 87, characterized by the fact that the treatment of the PC12 cells with the FGF leads these cells to a differentiation to a neuronal phenotype, showing the formation of axonal outgrowth and the migration of the EOPA localization from the perinucleus cytosol to the neurites extremities, suggesting a role for EOPA in the process of cellular extensions and inter-cellular connections formation.

Claim 91 - Process for determining the role of EOPA in the immunological processes, characterized by the fact that the size of the epitopes associated with the MHC class I is similar to that required to the peptide substrates/inhibitors to be susceptible to binding to EOPA (e.g., 7 to 13 amino acid residues).

Claim 92 - Process as described in the claim 91, characterized by the fact that the epitopes behave as competitive inhibitors of this enzyme, and/or as ligands which modulate the association of the EOPA with other cellular proteins; the opioid peptides and other bioactive peptides, presenting affinity to the EOPA might act in the same way, in the central nervous system as well as in the immune system, since the EOPA is also found in cells of the immune system, in particular, in macrophages and lymphocytes.

Claim 93 - Process as described in the claim 91, characterized by the fact that the expression of the MHC class I in nervous tissue is related to infectious and degenerative processes, and with the plasticity of the central nervous system; the control of the peptidase and the chaperon activities, or of the peptide receptor activity of EOPA, is directly related to the specific ligands (substrates, inhibitors and modulators) of the EOPA.

10 Claim 94 - Process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases of the central nervous system (CNS), wherein employing the sequence of the EOPA or its segments in the diagnostic methods for prevention and
15 treatment of these pathologies.

Claim 95 - Process as described in the claim 94, wherein based on the method for the identification of EOPA as described in the claim 61.

20 Claim 96 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological, psychiatric, and behavioral pathologies, and for the processes of tissue degeneration, characterized by the fact that specific ligands of the EOPA are employed in the prevention and the treatment of these diseases.

25 Claim 97 - Process as described in the claim 96, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55.

30 Claim 98 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological, psychiatric, and behavioral pathologies, and for the

processes of tissue degeneration, characterized by the fact that the inhibitors, antibodies and competitors compounds that interfere with the proteolytic, chaperon and/or soluble-receptor activities of the EOPA, may be used for
5 diagnosis, prevention and treatment of these diseases.

Claim 99 - Process as described in the claim 98, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and based on the process of the development of diagnosis methods, prevention
10 or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 100 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration,
15 characterized by the fact that the development of the EOPA inhibitors may be done by using the structural models based on natural toxins or compounds in general.

Claim 101 - Process as described in the claim 100, characterized by the fact that they are specially found in
20 the animal venoms.

Claim 102 - Process as described in the claims 100 and 101, wherein based on the process for identification of EOPA inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods,
25 prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 103 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration,
30 characterized by the fact that these compounds may be

employed in formulations or drugs which interfere in the process of neuropeptides inactivation or biotransformation by EOPA, besides of being used for the diagnosis, prevention and/or treatment of these diseases.

5 **Claim 104 - Process**, as described in the claim 103, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative
10 diseases as described in the claim 94.

Claim 105 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that the peptide inhibitors which
15 dislocate others peptides from the EOPA active-site may be employed.

Claim 106 - Process as described in the claim 105, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process
20 for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 107 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that it is possible to modulate the proteolytic/chaperona activity of EOPA by using the
25 inhibitors which interfere in the process of linfoproliferation or any other role in which the MHC class

I might be involved such as the infeccious, degenerative and plasticity of the central nervous system.

5 **Claim 108 - Process** as described in the claim 107, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

10 **Claim 109 - Process** for immunochemical and/or enzymatic diagnosis, for the prevention and for monitoring the evolution of pathologies, for the prognosis and/or treatment of congenital, infectious and degenerative pathologic conditions of the central nervous system, characterized by the fact that the proteolytic activity and
15 the interactions with other molecules properties of EOPA is regulated by inhibitors and/or substrates, or by other molecules which bind to EOPA, including the immunoglobulins.

20 **Claim 110 - Process** as described in the claim 109, characterized by the fact that the peptidase activity of EOPA can be determined by kinetic assays employing natural and synthetic peptide substrates.

25 **Claim 111 - Process** for the treatment of neurological, psychiatric and neurodegenerative pathologies, characterized by the fact that EOPA is related with the process of nuclear and neuronal migration, and also formation of the central nervous system, which occur during the embryogenesis.

30 **Claim 112 - Process** as described in the claim 111, characterized by the fact that the tissular and cellular

distribution of EOPA by immunohistochemistry shows the co-localization of EOPA and the opioid peptides and their precursors in the central nervous system, more precisely, in the cellular body and axons of the neurons.

- 5 **Claim 113 - Process**, as described in the claim 111, characterized by the fact that the EOPA distribution can be determined by using the "in situ" hybridization assays, which allowed observing a higher expression of EOPA in the brain.